The effects of manganese and barium on the cardiac pacemaker current, i_f, in rabbit sino-atrial node myocytes

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Received 12 July 1990; accepted 2 October 1990

Summary. The isolation of ionic fluxes contributing to electric currents through cell membranes often requires block of other undesired components which can be achieved, among others, by divalent cations. Mn^{2+} and Ba^{2+} are often used, for example, to block Ca and K currents. Here we have investigated the effects of these two cations on the properties of the hyperpolarization-activated pacemaker current i_f, in rabbit sino-atrial node myocytes, as obtained by voltage clamp analysis. We find that 2 mM Mn^{2+} shifts the i_f activation curve by 3.2 ± 0.3 mV towards more positive values. However, when 1 mM Ba^{2+} is also added, the positive shift is more than halved (1.3 ± 0.2 mV). We find, too, that in the absence of blocking cations the ACh-induced i_f inhibition is slightly higher than in their presence. These results indicate that the alteration of i_f kinetic properties by Ba^{2+} plus Mn^{2+}-containing solutions is minimal.
Key words. Pacemaker current; SA node; channel blockers.

Although the presence of i_f, a Na/K selective, inward current activated by hyperpolarization has been recognized in the mammalian SA node for more than a decade, its role in generating and modulating the normal diastolic depolarization remains controversial. A central part of this controversy concerns the voltage range of the pacemaker current i_f and its modification by neurotransmitters and divalent cations.

Often, divalent cations such as Ba^{2+} and Mn^{2+} are used to dissect the current i_f from interfering components (see for example DiFrancesco et al.[1]). Acting through surface-charge screening or binding, divalent cations may alter the position of the activation curve (see for example DiFrancesco and McNaughton[2], for the pacemaker current in Purkinje fibers).

Recently DiFrancesco et al.[3] have reported that low (nM) concentrations of acetylcholine shift i_f in a negative direction on the voltage axis and this effect of acetylcholine (ACh) is a major contributor to ACh's negative chronotropic effect. In their work, the dose-response relation for i_f inhibition by ACh was measured in solutions containing Ba^{2+} and Mn^{2+}. After that report appeared Brown et al.[5] suggested that the inclusion of Mn^{2+} in the bathing Tyrode can shift the activation curve for i_f as much as 10 mV in the positive direction on the voltage axis. This could affect the estimation of the relevance of i_f to the generation and control of the diastolic depolarization.

In the present report we have examined the effects of Mn^{2+}, and Mn^{2+} plus Ba^{2+} on i_f with the aim of exploring the extent of the modification induced by these cations on the current activation range.

Also we examined if Ba^{2+} or Mn^{2+} alter the dose response relation of ACh on i_f. We present data on the effects of ACh on the activation range of i_f in the absence of these blocking cations, at ACh concentrations which only minimally affect the ACh-activated K-current $i_{K,ACh}$ (up to 30 nM). Our results indicate that the presence of Ca-current blockers does not affect significantly the analysis of the properties of i_f, and in particular does not alter the dose-response curve of the i_f dependence on ACh.

Methods

The experiments were performed on acutely isolated SA node myocytes from the rabbit. The isolation procedure and electronic set up for whole cell voltage clamp have been described previously[1]. The cells were aliquotted into petri dishes and directly placed on the temperature controlled microscope stage for study. The Tyrode solution contained in mM 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 20 d-glucose and 5.0 Hepes NaOH (pH = 7.4). We added $BaCl_2$ (1 mM), $MnCl_2$ (2 mM) and acetylcholine chloride (3–30 nM) as indicated. The dialyzing solution in the pipette contained (in mM): 10 NaCl, 130 K aspartate, 2.0 Mg-adenosine triphosphate (ATP), 0.1 guanosine triphosphate (GTP), 1.0 EGTA, and 10 mM Hepes-KOH (pH = 7.2). External solutions were superfused from a pipette placed over the myocyte under study. Exchange took less than 1 s. The temperature was maintained at 35–36 °C. No corrections for liquid function potentials were applied in keeping with the previous study by DiFrancesco et al.[3]. Voltage shifts of the i_f activation curve under the action of shifting agents were measured as described in DiFrancesco et al.[3]. The holding potential was set to −35 mV in the control (Tyrode) solution which is above the top of the activation curve, and i_f was activated by a hyperpolarization to the mid-activation range applied every 2 seconds. In the presence of the shifting agent, the holding potential was adjusted manually to a new value by turning the holding potential knob (sensitivity = 0.1 mV), until the i_f time-course in the test solution overlapped that in the control solution. The displacement from −35 mV of the new holding potential repre-

sented the measured shift of the i_f activation curve. Given the high steepness of the i_f activation curve [1], this method allowed resolution of fractions of a mV.

Results

The effects of Mn^{2+} and Ba^{2+} on the voltage dependence of i_f. We examined the actions of 2 mM Mn^{2+} in the absence or presence of 1 mM Ba^{2+} on the voltage-dependent activation of the current i_f. Our first protocol consisted of holding the myocyte at −35 mV and hyperpolarizing the cell to a voltage within the i_f activation range. The holding potential of −35 mV was chosen because it is normally a few mV positive to the top of the i_f activation curve [1]. A sample set of results is illustrated in figure 1 A.

In this experiment the test potential was −85 mV, and the letters a–d indicate the order of solution application. Tyrode containing 2 mM Mn^{2+} increases the amplitude of i_f, but this increase was strongly reduced when 1 mM Ba^{2+} was also added. The effects of these solution changes were reversible.

A 3-pulse protocol [4] was then used to see if the alterations in i_f amplitude were due to a shift in the voltage dependence of i_f activation (fig. 1 B). The first hyperpolarizing pulse was delivered to the middle of the i_f activation curve and the second hyperpolarizing pulse was delivered to the bottom of the i_f activation curve. A third depolarizing pulse rapidly deactivated i_f before the cycle was repeated. A positive shift in i_f activation was indicated by a larger time-dependent current in response to the first hyperpolarizing step and a smaller time-dependent current in response to the second hyperpolarizing step. Figure 1 B illustrates that for Mn^{2+} (2 mM) + Ba^{2+} (1 mM) (*), and Mn^{2+} (2 mM) (x) a shift in i_f activation is observed. These results show that the action of the Ca-current blockers on i_f is essentially that of shifting the current activation range to more positive voltages.

We next examined quantitatively the action of the blocking cations on i_f by using the protocol shown in Figure 1 C. Here the test solution was superfused during a train of voltage steps of constant amplitude and the holding potential was then moved positive until the i_f current

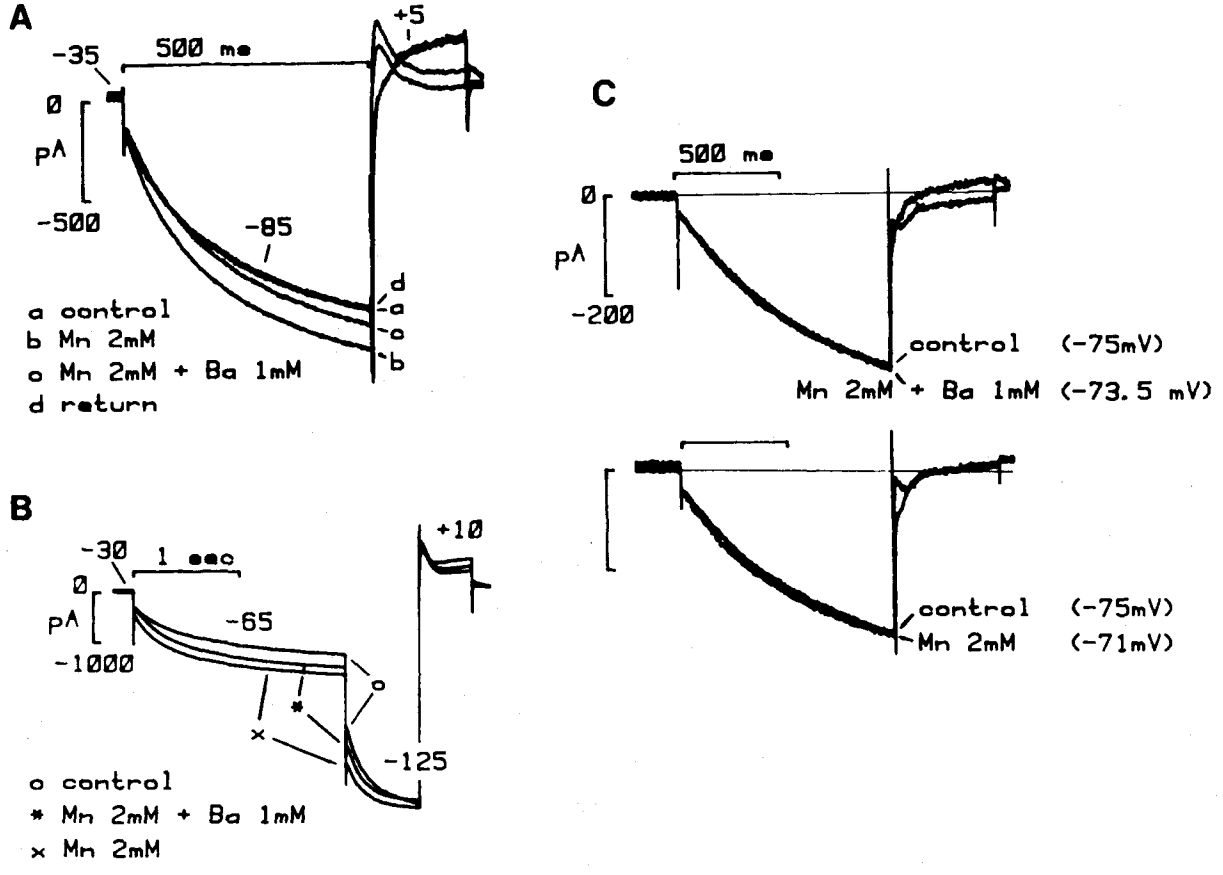

Figure 1. Action of Mn^{2+} 2 mM and of Mn^{2+} 2 mM plus Ba^{2+} 1 mM on the i_f kinetics. *A* i_f was activated by hyperpolarizing steps to −85 mV delivered every 2 s. Mn^{2+} and Mn^{2+} plus Ba^{2+} were added to the Tyrode solution according to the sequence indicated (a to c). The current increases in the presence of Mn^{2+}, but the increase is attenuated when also Ba^{2+} is added. Cell 2i-1. *B* A three-pulse protocol to the voltages indicated was applied every 2 s. Mn^{2+} increased i_f at −65 mV, and decreased it during the following step −125 mV, as expected from a positive shift of activation range. Further addition of Ba^{2+} partially reversed this effect. Cell 3i-4. *C* Measurement of shifts of i_f activation curve caused by either Mn^{2+} plus Ba^{2+} (upper) or Mn^{2+} alone (lower). Hyperpolarizing steps of fixed amplitude (40 mV) were applied from a holding potential of −35 mV in the control solution, and the holding potential was then moved positive, during superfusion with the test solution, until the i_f traces overlapped as shown. The shifts were + 1.5 mV for the Mn^{2+} plus Ba^{2+} solution, and + 4.0 mV for the Mn^{2+} solution. Cell 1f-6.

trace overlapped that in the control solution (see DiFrancesco et al.[3], fig. 2). This allowed an accurate estimation of the voltage shift of the i_f activation curve caused by Mn^{2+} or by Mn^{2+} plus Ba^{2+}. A sample set of data is provided in figure 1 C. In the upper panel a hyperpolarized level of −75 mV in the control solution elicits an equal activation of i_f as does a hyperpolarized level of −73 mV in Tyrode containing Mn^{2+} (2 mM) and Ba^{2+} (1 mM). This is equivalent to a 2 mV positive shift of the i_f activation curve. In the lower panel a similar protocol indicates that Mn^{2+} alone elicits a 4 mV positive shift in the i_f activation curve. For all myocytes examined by similar protocols we obtained a 3.16 mV ± 0.31 mV (SEM n = 14) positive shift of i_f activation when Mn^{2+} (2 mM) was added to the superfusing Tyrode. The shift was only 1.32 mV ± 0.18 mV (SEM n = 15) when Mn^{2+} (2 mM) and Ba^{2+} (1 mM) were included in the superfusate. These data indicate that 1) the shifting action of Mn^{2+} is smaller under our conditions than that reported by Brown et al.[5], and 2) the addition of Ba^{2+} minimizes the action of Mn^{2+}.

The effect of acetylcholine (3–30 nM) on the voltage dependence of i_f activation in the absence of Mn^{2+} and Ba^{2+}. Recently DiFrancesco et al.[3] have reported that low concentrations of acetylcholine shift i_f in the negative direction on the voltage axis. A comparison of this effect of acetylcholine with that on $i_{K,ACh}$ showed that the effect on i_f occurred at lower ACh concentrations. However, the dose-response relation for ACh effects on i_f was constructed in the presence of Ba^{2+} + Mn^{2+} to reduce contaminating currents while that for $i_{K,ACh}$ was constructed in the absence of these divalent cations. We therefore have repeated the experiments examining the concentration dependence of the ACh induced i_f activation curve shift in the absence of Ba^{2+} and Mn^{2+}. The results are plotted in figure 2.

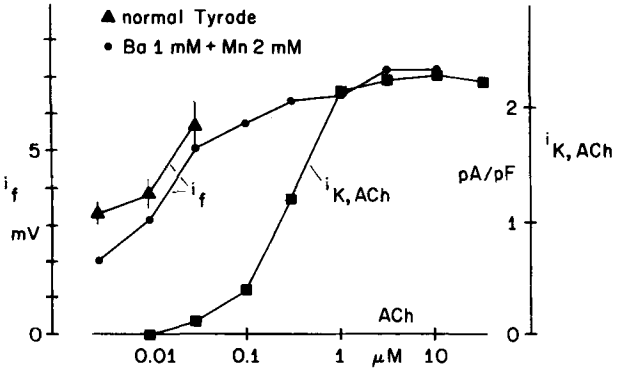

Figure 2. Dose-response relations for the shift of the i_f activation curve as a function of ACh concentration in normal Tyrode (▲) and in the presence of Mn^{2+} 2 mM plus Ba^{2+} 1 mM (●). A dose-response curve for i_K ACh (pA/pF) obtained in normal Tyrode (■) is also included. The two latter curves are redrawn from DiFrancesco et al.[3]. Data on i_f obtained in normal Tyrode (▲) are plotted as mean ± SEM from a total of 12 cells.

The protocol employed was identical to that of DiFrancesco et al.[3] and our figure 1 C. A hyperpolarizing pulse is applied from −35 mV in control solution to a potential within the i_f activation range. Acetylcholine at concentrations between 3 and 30 nM were superfused, and the shift in activation required for superposition of control and test i_f currents in response to hyperpolarization measured. This shift was −3.34 mV ± 0.31 mV (SEM n = 8) in 3 nM ACh, −3.87 mV ± 0.38 mV (SEM n = 6) at 10 nM ACh, and −5.7 mV ± 0.65 mV (SEM n = 6) at 30 nM ACh. These results are plotted in figure 2 along with the previous results of DiFrancesco et al.[3]. The shifts obtained in our experiments in normal Tyrode (▲) are similar to those obtained by DiFrancesco et al.[3] in the presence of Mn^{2+} and Ba^{2+} (●), and if anything are slightly larger. These results confirm the differences in the concentration dependence of i_f inhibition and $i_{K,ACh}$ activation by ACh.

Discussion

Our results indicate that Mn^{2+} (2 mM) shifts the i_f activation curve positive by about 3 mV on the voltage axis, and the combination of Mn^{2+} (2 mM) + Ba^{2+} (1 mM) shifts the i_f activation curve by less than half that amount. This result is surprising because increasing divalent cation concentrations should cause larger activation curve shifts through screening of membrane surface charges or binding to the external membrane surface. We are uncertain as to the reasons for this difference, but it could relate to the previously reported effects of Ba^{2+} alone to reduce the magnitude of i_f[1,6,7]. Recently Hagiwara and Irisawa[8] reported that decreasing internal pCa from 10 to 7 increased the amplitude of i_f and shifted i_f by 13 mV in the positive direction on the voltage axis. Brown et al.[5] suggest that the positive shift induced by Mn^{2+} was due to the ability of this ion to block the Na:Ca exchange[9] and induce a rise in sarcoplasmic $[Ca^{2+}]$. It is uncertain to what extent internal $[Ca^{2+}]$ can rise in our cells, since the patch pipette contained 1 mM EGTA. Whatever the mechanism, the magnitude of the shifts induced by these divalent cations are small and unlikely to distort to any significant degree the relationship between i_f activation and SA nodal pacemaker activity.

Furthermore, the dose-response relationship relating the shift in i_f activation and ACh, which could be distorted by an alteration of the apparent affinity of the receptor for ACh[10,11], is little affected by the divalent cations, as indicated in figure 2. The difference in affinity between ACh effects on i_f and ACh effects on the muscarinic induced K^+ current was not altered by removing the divalent cations present in the initial i_f experiments of DiFrancesco et al.[3].

In conclusion, i_f inhibition by low concentrations of ACh is independent of the presence of Mn^{2+} and Ba^{2+}. Previous results demonstrating that i_f activation starts as positive as −40 mV[12] are thus unlikely to be distorted due

to presence of Mn^{2+} and Ba^{2+}. The previous conclusions that i_f contributes to pacemaker activity in the SA node, and that ACh can exert its negative chronotropic effect through inhibition of i_f remain unaltered.

Acknowledgments. This work was supported by grants from the Ministero della P.I., the C.N.R. (grant CT 88-00491), the N.I.H. (grants HL35064, HL20558 and HL28958) and the MDA.

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0014-4754/91/050449-04$1.50 + 0.20/0

Effect of pancreatic secretions upon ileal disaccharidase activities of neonatal miniature pigs

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Received 17 May 1990; accepted 8 October 1990

Summary. Mechanisms by which pancreatic secretions influence disaccharidase activities in the distal small intestine have been investigated in 1-week-old miniature pigs. Using a combination of biochemical, cytochemical and morphological techniques it has been found that the decrease in lactase specific activity is due solely to a reduction in villus surface area. By contrast, the increased sucrase-isomaltase activities, which occur despite the reduction in villus surface area, are due entirely to increased enzyme expression during enterocyte differentiation.
Key words. Disaccharidases; pancreatic proteases; cellular and structural adaptation; small intestine.

Expression of intestinal disaccharidases [1] and the secretion of pancreatic enzymes [2] in the pig are age dependent. The latter have been shown to affect disaccharidase activities of the adult small intestine through proteolysis of the enzymes concerned [3]. There is, on the other hand, evidence to suggest that pancreatic secretions also exert trophic effects in the small intestine [4] and this could increase the villus capacity to hydrolyze sugars. Changes in intestinal activities of disaccharidases along the length of the tract have recently been partitioned into components dependent upon enterocyte development and villus structure but it is not known whether these changes are due to local effects produced by pancreatic secretions [5]. The purpose of the present study was to estimate the relative importance of these two separate influences on ileal disaccharidase activities in the neonatal intestine after the elimination of pancreatic secretions from the lumen by ligation of the pancreatic duct.

Methods

Seven pairs of 1-week-old Hanford miniature pigs (Charles River Breeding Laboratories, Wilmington, MA) underwent either sham (S) or pancreatic duct ligation (PDL) operations. This procedure does not interfere with the bile duct since these ducts are discrete entities in the pig. All pigs received a prophylactic dose of 50 mg cefotaxime/kg b. wt prior to surgery. Pigs were anesthetized by i.m. injections of 0.03 mg fentanyl/kg followed 15 min later by 8 mg ketamine/kg b. wt. The abdomen was opened and the pancreatic duct ligated using 3-0 suture. Sham-operated animals underwent identical surgery except that ligation of the duct was omitted. The abdomen was then irrigated with 10 ml of sterile normal saline and closed. After the pigs had recovered they were returned to the same cage and maintained on a standard swine weaning formula (Soweena, Merrick's, Union Center, WI). All pigs received formula prepared from the same batch and were allowed free access to food which was provided fresh three times daily. Throughout the study, it was noted that all animals were feeding. Percentage composition of dry formula was 25% protein, 10% fat, 50% lactose and the remainder being inorganic solid and vitamins. At 14 days of age the animals were killed by a lethal i.m. injection of 0.05 mg fentanyl and 11.0 mg ketamine/kg b. wt, and samples of the distal small intestine were taken. Disaccharidase activities were determined both biochemically and cytochemically as described previously [1]. Villus surface area was determined